

The single kinin receptor signals to separate and independent physiological pathways in Malpighian tubules of the yellow fever mosquito.

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14. ABSTRACT <p>In the past we have used the kinins of the cockroach <i>Leucophaea</i> (the leucokinins) to evaluate the mechanism of diuretic action of kinin peptides in Malpighian tubules of the yellow fever mosquito <i>Aedes aegypti</i>. Now using the kinins of <i>Aedes</i> (the aedeskinins), we have found that in isolated <i>Aedes</i> Malpighian tubules all three aedeskinins (1&#956;M) significantly 1) increased the rate of fluid secretion (&#56257;&#57144;&#56258;&#57318; &#56260;&#56886), 2) hyperpolarized the basolateral membrane voltage (Vbl), and 3) decreased the input resistance (Rin) of principal cells, consistent with the known increase in the Cl⁻ conductance of the paracellular pathway in <i>Aedes</i> Malpighian tubules. Aedeskinin-III studied in further detail, significantly increased &#56257;&#57144;&#56258;&#57318; &#56260;&#56886 with an EC₅₀ of 1.5 x10⁻⁸ M. In parallel, the Na⁺ concentration in secreted fluid significantly decreased, and the K⁺ concentration significantly increased. The concentration of Cl⁻ remained unchanged. While the three aedeskinins triggered effects on Vbl, Rin and &#56257;&#57144;&#56258;&#57318; &#56260;&#56886 , synthetic kinin analogs (which contain modifications of the C-terminal amide pentapeptide core sequence critical for biological activity) displayed variable effects. For example, kinin analog 1578 significantly stimulated &#56257;&#57144;&#56258;&#57318; &#56260;&#56886 but had no effect on Vbl and Rin, whereas kinin analog 1708 had no effect on &#56257;&#57144;&#56258;&#57318; &#56260;&#56886 but significantly affected Vbl and Rin. These observations suggest separate signaling pathways activated by kinins. One triggers the electrophysiological response, and the other triggers fluid secretion. It remains to be determined whether the two signaling pathways emanate from a single kinin receptor via agonist-directed signaling or from a differentially glycosylated receptor. Occasionally, Malpighian tubule did not exhibit a detectable response to natural and synthetic kinins. Hypothetically, the expression of the kinin receptor may depend on developmental, nutritional, and/or reproductive signals.</p>		
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Abstract

In the past we have used the kinins of the cockroach *Leucophaea* (the leucokinins) to evaluate the mechanism of diuretic action of kinin peptides in Malpighian tubules of the yellow fever mosquito *Aedes aegypti*. Now using the kinins of *Aedes* (the aedeskinins), we have found that in isolated *Aedes* Malpighian tubules all three aedeskinins (1 μ M) significantly 1) increased the rate of fluid secretion (\dot{V}_s), 2) hyperpolarized the basolateral membrane voltage (V_{bl}), and 3) decreased the input resistance (R_{in}) of principal cells, consistent with the known increase in the Cl^- conductance of the paracellular pathway in *Aedes* Malpighian tubules. Aedeskinin-III, studied in further detail, significantly increased \dot{V}_s with an EC_{50} of 1.5×10^{-8} M. In parallel, the Na^+ concentration in secreted fluid significantly decreased, and the K^+ concentration significantly increased. The concentration of Cl^- remained unchanged. While the three aedeskinins triggered effects on V_{bl} , R_{in} and \dot{V}_s , synthetic kinin analogs (which contain modifications of the C-terminal amide pentapeptide core sequence critical for biological activity) displayed variable effects. For example, kinin analog 1578 significantly stimulated \dot{V}_s but had no effect on V_{bl} and R_{in} , whereas kinin analog 1708 had no effect on \dot{V}_s but significantly affected V_{bl} and R_{in} . These observations suggest separate signaling pathways activated by kinins. One triggers the electrophysiological response, and the other triggers fluid secretion. It remains to be determined whether the two signaling pathways emanate from a single kinin receptor via agonist-directed signaling or from a differentially glycosylated receptor. Occasionally, Malpighian tubule did not exhibit a detectable response to natural and synthetic kinins. Hypothetically, the expression of the kinin receptor may depend on developmental, nutritional, and/or reproductive signals.

Key words: *Aedes aegypti*, Malpighian tubules, principal cells, aedeskinin, G protein-coupled kinin receptor (GPCR), Ramsay fluid secretion assay, membrane voltage, input resistance, principal cells, α - and β -analogs of insect kinins, functionally selective agonists.

Introduction

Epithelial secretion and absorption are the two mechanisms for regulating the volume and composition of the hemolymph in insects (4, 16). In the case of Malpighian tubules, epithelial secretion delivers a fluid to the lumen in upstream (distal) segments of the Malpighian tubule creating a urinary space into which excess solutes, metabolic wastes and toxins can subsequently be dumped for excretion. In downstream (proximal) segments of the tubule, in the hindgut and the rectum, reabsorptive mechanisms recover the electrolytes and water that initially served to produce the urinary space while concentrating unwanted solutes for excretion.

The transport mechanisms of epithelial secretion and absorption are under hormonal control as Malpighian tubules execute the tasks of extracellular fluid homeostasis (4, 20). Diuretic hormones stimulate tubular secretion and urine flow, whereas antidiuretic hormones stimulate tubular reabsorption and decrease the excretion of urine.

Four families of diuretic peptides have been identified in insects: the CRF-like peptides, the calcitonin-like peptides, the CAPA peptides and the kinin peptides (20, 22, 26). Holman, Cook and Nachman isolated the first kinin peptides from head extracts of the cockroach *Leucophaea maderae* using the motility of the cockroach hindgut as a bioassay. Thus, the isolated peptides were considered smooth muscle stimulants, i.e. myokinins, and called leucokinins (30).

Agonists of smooth muscle are often secretagogues which led our laboratory to examine the effect of leucokinin on fluid secretion in Malpighian tubules of the yellow fever mosquito, *Aedes aegypti* (27). We found that leucokinin-VIII from the cockroach stimulated fluid secretion in *Aedes* Malpighian tubules, suggesting the wide distribution of kinins in insects. Indeed, the stimulation of fluid secretion by leucokinin and other kinins has now been observed in Malpighian tubules of the house cricket (23), locust (21, 55), corn earworm (10), fruit fly (36, 54), and housefly (32). The insect subclass Pterygota, the winged insects, includes 97% of all insects, and the kinins have been found in every order of this subclass that has been examined for kinin peptides.

In the past we have used the kinins of the cockroach (the leucokinins) to elucidate the mechanism of kinin action in Malpighian tubules of the yellow fever mosquito (5, 27, 39, 65-67). As early as 1994, the laboratory of Veenstra has identified three kinins of the mosquito *Aedes aegypti*, the aedeskinins (57). In his laboratory, all three aedeskinins depolarized the

transepithelial voltage in *Aedes* Malpighian tubules like leucokinin (58). However, only aedeskinin-I and -III stimulated fluid secretion in his laboratory, whereas aedeskinin-II had no detectable effect on fluid secretion (58).

In contrast to Veenstra's earlier work, we report here that all three aedeskinins (including aedeskinin-II) significantly increase the rate of transepithelial fluid secretion. Moreover, aedeskinin-III increases the transepithelial secretion of NaCl and KCl, like leucokinin-VIII (5, 39). Furthermore, all three aedeskinins hyperpolarize the basolateral membrane voltage of principal cells (V_{bl}) while decreasing the input resistance of principal cells (R_{in}), like leucokinin-VIII (5, 39). Thus, aedeskinins and leucokinins share similar if not the same mechanisms of action.

Our study of five additional synthetic kinin analogs that possess single or multiple substitutions of β - and/or α,α -disubstituted amino acids both within and just outside the C-terminal pentapeptide core sequence critical for biological activity, reveal separate and independent signaling pathways that mediate the effects on fluid secretion (\dot{V}_s) and electrophysiology (V_{bl} , R_{in}). Some kinin analogs increase \dot{V}_s without affecting V_{bl} and R_{in} . Other analogs show the opposite: no effect on \dot{V}_s but clear effects on V_{bl} and R_{in} . Since the Malpighian tubule of *Aedes aegypti* is thought to express only one kinin receptor (42), different physiological effects that originate from a single receptor may reflect mechanisms of ligand-dependent signaling and/or different glycosylation states of the receptor. Thus, the G protein-coupled kinin receptor is functionally more diverse than previously assumed.

Lastly, we find that some tubules do not respond to aedeskinins at all (natural or the analogs). Preliminary observations point to differences in individual mosquitoes and to differences in populations of mosquitoes.

Materials and Methods

Mosquitoes and Malpighian tubules. The method for rearing mosquitoes (*Aedes aegypti*) is described in (39) with the exception that we now feed larval mosquitoes Tetramin® flakes (tropical fish food) finely ground with mortar and pestle. On the day of the experiment a female mosquito, 4-7 days post-eclosion, was cold anesthetized and decapitated. Malpighian tubules were removed from the mosquito under Ringer solution by pulling gently on the rectum which freed the gut and Malpighian tubules from the abdominal cavity. A Malpighian tubule was then removed with forceps from its attachment to the gut. The proximal end of the tubule (crushed by the forceps) was subsequently teased open with sharp jeweler's broaches.

Ringer solution and diuretic peptides. Ringer solution contained the following (in mM): 150 NaCl, 3.4 KCl, 25 HEPES, 1.8 NaHCO₃, 1.0 MgSO₄ (or MgCl₂), 1.7 CaCl₂, and 5.0 glucose. The pH was adjusted to 7.1 using 1M NaOH. Table 1 lists the peptides used in the present study. Leucokinin-VIII, aedeskinins and kinin analogs were synthesized in the laboratory of Nachman (50, 69). The identity of each peptide was confirmed by MALDI-TOF mass spectrometry, and the amount quantified by amino acid analysis. The three aedeskinins are splice variants of the same gene (58).

In one set of experiments we dissolved the peptides directly in Ringer solution and stored them at -20 °C until use. In another set of experiments the peptides were dissolved in 80% acetonitrile and 0.01% TFA and stored at 4 °C. On the day of the experiment an aliquot was lyophilized and then dissolved in Ringer solution.

The Ramsay method of fluid secretion. The rate of fluid secretion (\dot{V}_s) was measured at room temperature in isolated Malpighian tubules as developed by Ramsay (45) and adopted by us (29). In brief, the distal (blind) end of the tubule was bathed in a Ringer droplet of 50 μ l under light mineral oil. The open end of the tubule was pulled into the oil with a glass hook so that fluid secreted by the tubule exited into the oil (Fig. 1a). The glass hook was formed on a microforge (Stoelting Co., Wood Dale, IL) using soft glass (R-6, Drummond Scientific Co., Broomall, PA). After acid-washing the glass hook with a mixture of K₂CrO₄ and H₂SO₄, the hook was exposed to the vapor of 20 μ l dimethyl-dichlorosilan for 90 s (Fluka, Buchs, Switzerland) and then baked

overnight at 110 °C. The silanization prevented secreted fluid from spreading out along the hook.

Each tubule was used as its own control. The initial 30 min marked the control fluid secretion period. Thereafter, the control droplet (nanoliter volumes, 10^{-9} liter) was removed and prepared for the analysis of its ionic composition with the electron probe. Subsequently, 5 μ l of Ringer solution was removed from the peritubular bath and replaced with 5 μ l of Ringer solution containing one of the peptides of Table 1.

The 30 min experimental period began as soon as the peptide had been added to the peritubular bath. Rates of fluid secretion were determined by plotting cumulative volume (nl) secreted by the tubule as a function of time (30 min), each for the control and the experimental period. The plots were usually linear, yielding the rate of fluid secretion as the slope of the line (Microsoft Excel 2007, Microsoft, Redmond, WA, USA).

Electron probe analysis of fluid electrolyte composition. The ionic composition of fluid secreted by Malpighian tubules was analyzed with the electron probe as described previously (7, 62), except that we used the JEOL JXA 8900 EPMA Microprobe (Tokyo, Japan). Control and experimental fluid droplets collected in the Ramsay assay were prepared for electron probe analysis within 1 hour of collection. A picoliter pipette was used to deposit a constant volume of unknowns (secreted fluid droplets) and standard solutions of known Na^+ , K^+ , and Cl^- concentrations onto a polished beryllium block under light mineral oil.

Volumetric picoliter (10^{-12} liter) pipettes were produced using soft glass (R6, Drummond Scientific Co., Broomall, PA) and a microforge (Stoelting Co., Wood Dale, IL) to form a volumetric chamber of approximately 60 pl from the pipette opening to an internal constriction. The exact pipette volume is not important as long as the same pipette is used for both standards and unknowns. Prior to use, the volumetric part of the pipette was vapor-silanized as described above. The silanization prevents fluid from sticking to the walls of the volumetric chamber.

After standards and unknowns were deposited on the beryllium block under oil (using the same picoliter pipette), the oil was removed in three washes with chloroform. The chloroform wash also removes water from the droplets, leaving salt crystals behind. The block is then stored in a desiccator under vacuum until analysis with the electron probe.

Wavelength dispersive spectroscopy is based on X-Ray emissions specific to each element in the sample. The JEOL JXA 8900 EPMA Microprobe allows the measurement of as many as 3 elements in a single sample: Na^+ , K^+ , and Cl^- in the present study. Seven standard solutions containing a range of Na^+ , K^+ , and Cl^- concentrations were used to produce standard curves. X-Ray counts from the unknowns (secreted fluid droplets) were read against the appropriate standard curve to yield the Na^+ , K^+ , and Cl^- concentration in secreted fluid. Transepithelial secretion rates of Na^+ , K^+ , and Cl^- were calculated as the product of the ion concentration and the fluid secretion rate for both control and stimulated conditions.

Electrophysiological effects of aedeskinin and analogs. The basolateral membrane voltage (V_{bl}) and the input resistance (R_{in}) of a principal cell of an isolated Malpighian tubule were measured as shown in Fig. 1b and as described previously (8, 34). In brief, Malpighian tubules were isolated and deposited on a sheet of Parafilm® (American National Can, Menasha, WI) that had been stretched over the bottom of the Ringer perfusion bath of 0.5 ml volume. Malpighian tubules stick to stretched Parafilm® which secures them in place for the impalement of principal cells with microelectrodes and for flushing the Ringer bath in the wash-out of test substances.

Microelectrodes (Omega dot borosilicate glass capillaries, 30-30-1; Frederick Haer & Co, St Bowdoinham, ME or 1B100F-4, World Precision Instruments, Inc., Sarasota, FL) were pulled on a programmable puller (Model P-97; Sutter Instruments, Novato, CA) to yield resistances between 10 M Ω and 40 M Ω when filled with 3 M KCl. The microelectrode was bridged to the measuring hardware using an Ag/AgCl junction. The junction was prepared by first degreasing a silver wire (O.D. 0.25 mm) with alcohol, and then by Cl^- plating it in either 1) 0.1 M HCl for 20 min at a current of 50 μA , or 2) in a concentrated solution of household Clorox for 15 min. The Ag/AgCl wires were inserted directly into the back of voltage and current electrodes (Fig. 1b). An Ag/AgCl wire with an O.D. of 0.5 mm was inserted into a 4% agar bridge of Ringer solution to serve as the ground electrode.

V_{bl} was measured with a Geneclamp 500 electrometer (Axon Instruments, Molecular Devices, Sunnyvale, CA) at room temperature. The input resistance of the impaled principal cell was measured from current-voltage plots generated by voltage-clamping the cell in a series of 5 increasing voltage clamp steps: $\Delta 5$ mV, 400 ms each, starting at $V_{bl} - 10$ mV. The voltage-stepping protocol and data acquisition were executed digitally using a Digidata 1440 (Molecular

Devices) under control of the Clampex module of the pCLAMP software package (Version 10, Molecular Devices).

In the typical experiment, a single principal cell near the blind (distal) end of the Malpighian tubule was impaled with both voltage and current electrodes (Fig. 1b). The experiment was continued if both electrodes measured values of V_{bl} within 10 mV of each other. After recording a stable V_{bl} and R_{in} , the kinin of interest was added to the peritubular Ringer solution to yield a concentration of 10^{-6} M. New steady state values of V_{bl} and R_{in} were recorded 1-2 min later as shown in Figs. 2, 4 and 5. The kinin was then washed out by flushing the 0.5 ml Ringer bath with Ringer solution at a rate of 5 ml/min.

Statistical treatment of data.

Statistical significance of the data was evaluated using the paired Student t-test where each tubule was used as its own control. To construct a dose response curve, the data were fitted using a sigmoid dose-response regression offered by the GraphPad Prism version 4.0 software for Windows (GraphPad Software, San Diego CA, USA).

Results

The effect of leucokinin-VIII on transepithelial fluid secretion and tubule electrophysiology.

From a previous study (39) we know that leucokinin-VIII significantly increases the rate of fluid secretion in isolated Malpighian tubules from 0.49 ± 0.04 nl/min to 0.91 ± 0.08 nl/min ($p < 0.0001$, $n = 10$ tubules). In the present study, leucokinin-VIII also had electrophysiological effects on principal cells of the tubule. In the representative experiment shown in Fig. 2, leucokinin-VIII hyperpolarized V_{bl} from about -90 mV to -108 mV while lowering R_{in} from 516 k Ω to 330 k Ω . Both effects are reversible upon washout of leucokinin-VIII from the peritubular bath.

The effect of aedeskinins on transepithelial fluid secretion and tubule electrophysiology. All three aedeskinins tested at a concentration of 10^{-6} M significantly 1) increased the rate of fluid secretion (\dot{V}_s), 2) hyperpolarized the basolateral membrane voltage (V_{bl}) and decreased the input resistance (R_{in}) of principal cells in isolated *Aedes* Malpighian tubules (Fig. 2).

Aedeskinin-I significantly increased \dot{V}_s from 0.53 ± 0.06 nl/min to 0.84 ± 0.11 nl/min in 21 Malpighian tubules ($p < 0.05$). In electrophysiological studies, the addition of aedeskinin-I to the peritubular medium of 14 Malpighian tubules immediately and significantly hyperpolarized V_{bl} from -61.2 ± 6.6 mV to -69.7 ± 7.2 mV ($p < 0.001$, paired t-test) and significantly decreased R_{in} from 413.0 ± 91.6 k Ω to 326.4 ± 75.3 k Ω ($p < 0.05$, paired t-test). A representative experiment is shown in Fig. 2. The effects on V_{bl} and R_{in} were reversible upon washout of aedeskinin-I (data not shown).

Aedeskinin-II significantly increased \dot{V}_s from 0.51 ± 0.07 nl/min to 0.93 ± 0.10 nl/min in 21 Malpighian tubules ($p < 0.001$). In electrophysiological studies, the peritubular addition of aedeskinin-II immediately and significantly hyperpolarized V_{bl} from -70.2 ± 8.1 mV to -77.7 ± 8.2 mV in 10 principal cells of as many tubules ($p < 0.001$, paired t-test). In parallel, the input resistance dropped significantly from 331.4 ± 41.2 k Ω to 307.0 ± 38.7 k Ω ($p < 0.05$, paired t-test). A representative experiment is shown in Fig. 2. The effects on V_{bl} and R_{in} were reversible upon washout of aedeskinin-II.

Aedeskinin-III significantly increased \dot{V}_s from 0.52 ± 0.06 nl/min to 1.04 ± 0.13 nl/min in 21 Malpighian tubules ($p < 0.001$, paired t-test). In electrophysiological studies, the peritubular

addition of aedeskinin-III immediately and significantly hyperpolarized V_{bl} from -64.3 ± 3.7 mV to -87.8 ± 4.3 mV in 13 principal cells of as many tubules ($p < 0.001$, paired t-test). In parallel, the input resistance dropped significantly from 343.3 ± 54.8 k Ω to 265.2 ± 41.9 k Ω ($p < 0.01$, paired t-test). A representative experiment is shown in Fig. 2. The effects on V_{bl} and R_{in} were reversible upon washout of aedeskinin-III (data not shown).

Invariably, if a principal cell exhibited spontaneous oscillation of V_{bl} under control conditions, then the peritubular addition of aedeskinin-I, -II, or -III substantially diminished or eliminated entirely these oscillations (Fig. 2).

Aedeskinin-III and transepithelial fluid secretion: a dose response study. To explore the diuretic mechanism of action of the aedeskinins, we studied in detail the effects of aedeskinin-III on rates of transepithelial fluid secretion (\dot{V}_s) in isolated *Aedes* Malpighian tubules. Aedeskinin-III was selected for this study because it was the most potent secretagogue in our laboratory (Fig. 2) and in the laboratory of Veenstra (58).

Fig. 3a illustrates a dose response to aedeskinin-III. At a concentration of 10^{-10} M, aedeskinin-III had no effect on \dot{V}_s . At concentrations 10^{-8} M and higher, aedeskinin-III significantly increased \dot{V}_s . Maximal stimulation was observed at 10^{-6} M with a 104.50 ± 14.01 % increase in \dot{V}_s in 21 tubules ($p < 0.001$, paired t-test) and an EC_{50} value of 1.5×10^{-8} M (Fig. 3a). The average control rate of \dot{V}_s in all 71 Malpighian tubules shown in Fig. 3a was 0.58 ± 0.04 nl/min.

The effect of aedeskinin-III on transepithelial Na^+ , K^+ , and Cl^- secretion was tested in 10 separate Malpighian tubules (Fig. 3b,c). In these tubules, the average control rate of fluid secretion was 0.48 ± 0.10 nl/min (data not shown). It increased significantly to 0.93 ± 0.21 nl/min in the presence of 10^{-6} M aedeskinin-III ($p < 0.01$, paired t-test). In the same 10 tubules, aedeskinin III significantly decreased the concentration of Na^+ in secreted fluid from 153.3 ± 13.1 mM to 114.3 ± 12.8 mM ($p < 0.01$, paired t-test), and significantly increased the concentration of K^+ from 61.9 ± 7.5 mM to 83.9 ± 6.4 mM ($p < 0.02$, paired t-test). The change in Cl^- concentration from 183.7 ± 13.2 mM to 172.1 ± 6.9 mM was not significant.

The product of the fluid secretion rate and the ion concentration in secreted fluid yields the rate of transepithelial ion secretion. As shown in Fig. 3c, aedeskinin-III significantly increased the tubular secretion rates of Na^+ , K^+ , and Cl^- . The transepithelial Na^+ secretion increased 32%,

from 70.9 ± 14.8 pmol/min to 93.4 ± 18.9 pmol/min ($p < 0.02$, paired t-test). The secretion of K^+ increased 140%, from 33.5 ± 8.8 pmol/min to 80.5 ± 20.5 pmol/min ($p < 0.01$, paired t-test), and the secretion of Cl^- increased 69%, from 94.1 ± 20.6 pmol/min to 158.7 ± 34.3 pmol/min ($p < 0.01$, paired t-test).

Effects of synthetic kinin analogs on fluid secretion and tubule electrophysiology.

1460 II. In the Ramsay fluid secretion assay, analog 1460 II (10^{-6} M) significantly increased \dot{V}_s from 0.76 ± 0.11 nl/min to 1.32 ± 0.24 nl/min in the 6 tubules studied ($p < 0.01$, paired t-test; Fig. 4). In the electrophysiological assay, analog 1460 II (10^{-6} M) significantly and reversibly hyperpolarized the basolateral membrane voltage V_{bl} from -70.0 ± 7.7 mV to -84.4 ± 6.2 mV in 8 principal cells from as many tubules ($p < 0.01$, paired t-test). In parallel, the input resistance (R_{in}) decreased significantly and reversibly from 323.0 ± 42.9 k Ω to 281.5 ± 34.3 k Ω ($p < 0.01$, paired t-test). The representative experiment shown in Fig. 4 illustrates V_{bl} oscillating between -65 and -85 mV under control conditions. The addition of analog 1460 II to the peritubular medium hyperpolarized V_{bl} to a steady state voltage of -98 mV; in parallel the input resistance of the principal cell dropped from 208 k Ω to 169 k Ω . Upon washout of 1460 II, V_{bl} returned to -74 mV while R_{in} increased to 255 k Ω .

1547. In the Ramsay fluid secretion assay, analog 1547 (10^{-6} M) significantly increased \dot{V}_s from 0.47 ± 0.07 nl/min to 1.01 ± 0.20 nl/min in the 7 tubules studied ($p < 0.01$, paired t-test; Fig. 4). In the electrophysiological assay, analog 1547 significantly increased V_{bl} from -73.2 ± 6.3 mV to -83.0 ± 7.2 mV in 9 principal cells from as many tubules ($p < 0.01$, paired t-test), but the effects on input resistance were variable. The input resistance increased by as much as 131 k Ω in 5 tubules, but decreased by as much as 209 k Ω in 4 tubules even though all 9 tubules recorded the hyperpolarization of V_{bl} . Thus, the change in mean input resistance from 337.3 ± 23.4 k Ω to 305.5 ± 58.6 k Ω was not significant. In the experiment shown in Fig.4, analog 1547 reversibly hyperpolarized V_{bl} together with a drop in R_{in} .

1577. In the Ramsay fluid secretion assay, analog 1577 (10^{-6} M) significantly increased \dot{V}_s from 0.72 ± 0.18 nl/min to 1.22 ± 0.22 nl/min in the 6 tubules studied ($p < 0.05$, paired t-test; Fig. 4). In the electrophysiological assay, analog 1577 significantly hyperpolarized V_{bl} from -73.8 ± 2.8 mV to -85.9 ± 2.3 mV in 8 principal cells from as many tubules ($p < 0.01$, paired t-test) but the effects on input resistance were variable. In 6 of 8 tubules the R_{in} decreased in the

presence of analog 1577, and in 2 tubules R_{in} increased. Thus, the small decrease in the mean input resistance from $372.4 \pm 26.7 \text{ k}\Omega$ to $356.2 \pm 26.0 \text{ k}\Omega$ did not reach statistical significance. In the experiment shown in Fig. 4, analog 1577 hyperpolarized V_{bl} from -62 mV to -75 mV ; in parallel the input resistance (R_{in}) increased from $693 \text{ k}\Omega$ to $759 \text{ k}\Omega$.

1578. In the Ramsay fluid secretion assay, analog 1578 (10^{-6} M) significantly increased \dot{V}_s from $0.49 \pm 0.05 \text{ nl/min}$ to $1.01 \pm 0.16 \text{ nl/min}$ in the 6 tubules studied ($p < 0.01$, paired t-test; Fig. 4). However, in the electrophysiological assay, analog 1578 (10^{-6} M) had no effect on V_{bl} and R_{in} . As shown in the representative experiment of Fig. 4, the usual step change in V_{bl} and R_{in} upon the addition of aedeskinins did not occur after adding analog 1578 to the peritubular bath. As such, the small change in the mean V_{bl} from $-81.1 \pm 5.9 \text{ mV}$ to $-84.1 \pm 6.1 \text{ mV}$ in 10 principal cells from as many tubules was not statistically significant. Likewise, the small drop in mean R_{in} from $401.2 \pm 45.8 \text{ k}\Omega$ to $388.4 \pm 47.4 \text{ k}\Omega$ was not significant.

We point out that tubules which did not respond to analog 1578 still responded to aedeskinin-III (and/or to leucokinin-VIII) at concentrations of 10^{-6} M (Fig. 5e). Thus, analog 1578 was remarkable in that it clearly stimulated fluid secretion in *Aedes* Malpighian tubules without effects on tubule electrophysiology (Fig. 4).

1708. In the Ramsay fluid secretion assay, analog 1708 (10^{-6} M) is the only one that had no effect on the mean rate of fluid secretion in 29 tubules studies (Fig. 4), which stems from the fact that in 12 tubules analog 1708 increased \dot{V}_s , whereas in 17 others it decreased \dot{V}_s . In contrast, analog 1708 significantly hyperpolarized V_{bl} from $-73.3 \pm 4.6 \text{ mV}$ to $-95.0 \pm 5.0 \text{ mV}$ in 9 principal cells from as many tubules ($p < 0.001$, paired t-test) and significantly decreased R_{in} from $439.1 \pm 60.0 \text{ k}\Omega$ to $351.1 \pm 38.1 \text{ k}\Omega$ ($p < 0.02$, paired t-test). In the representative experiment shown in Fig. 4, analog 1708 hyperpolarized V_{bl} from -92 mV to -110 mV while decreasing R_{in} from $292 \text{ k}\Omega$ to $274 \text{ k}\Omega$. In this and another tubule experiment, the effects of analog 1708 on V_{bl} and R_{in} were irreversible upon washout of 1708. In the remaining 7 tubules, the effects on principal cell voltage and resistance were slowly reversible upon washout of 1708. Thus, analog 1708 was remarkable in that it clearly affected the electrophysiology of *Aedes* Malpighian tubules without consistent effects on fluid secretion (Fig. 4).

Spontaneous voltage oscillations. Malpighian tubules of *Aedes aegypti* often display oscillating membrane voltages under control, unstimulated conditions (Figs. 2,4,5). From previous studies

we know that oscillations of the basolateral membrane voltage (V_{bl}) can come about from oscillating changes in the paracellular Cl^- conductance (6, 8). As the paracellular Cl^- conductance increases, V_{bl} hyperpolarizes.

The natural aedeskinins and those synthetic kinin analogs with effects on tubule electrophysiology not only caused the hyperpolarization of V_{bl} together with the drop in R_{in} (Figs. 2,4), they also substantially diminished or abolished the oscillations of V_{bl} . Upon washing out the kinin, the oscillations usually reappeared.

Variability in the response to kinins. We make the occasional observation that Malpighian tubules do not respond to kinins, neither the natural aedeskinins nor the synthetic analogs (Fig. 5). We encounter non-responding tubules under three conditions. First, Malpighian tubules with control values of V_{bl} more negative than -75 mV and non-oscillating, are less likely to respond to kinins than tubules with the usual control values of V_{bl} in the vicinity of -60 mV. In the example shown in Fig. 5b, V_{bl} is about -80 mV, and it does not oscillate. Aedeskinin-I (10^{-6} M) had no effect on V_{bl} and R_{in} in this tubule. Since kinins hyperpolarize V_{bl} , we hypothesize that the tubule was already kinin-activated in the mosquito from which it had been isolated. Second, the failure to respond to kinins is also observed in tubules with low and oscillating values of V_{bl} . In the example illustrated in Fig. 5c, V_{bl} oscillates around values of -50 mV. The tubule did not respond to aedeskinin-I. Third, we observe receptor desensitization in the presence of kinins. As shown in Fig. 5d, the isolated Malpighian tubule displayed the typical electrophysiological response in the presence of kinin: 1) the hyperpolarization of the basolateral membrane voltage, 2) the drop in the cell input resistance, and 3) the cessation of V_{bl} oscillations. However, after 5 min in the presence of aedeskinin-III, V_{bl} started to repolarize, and voltage oscillations returned after about 10 min. As V_{bl} continued to repolarize and as voltage oscillations increased with time, the cell input resistance returned to control values in the presence of aedeskinin-III. The return to control values in the presence of agonist indicates receptor desensitization.

To rule out the study of non-responding tubules when a kinin analog had no effect, we routinely pulsed the tubule with aedeskinin-III. For example, as shown in Fig. 5e, the tubule responded to aedeskinin-III but not to kinin agonist 1578.

Discussion

Aedeskinin-II stimulates fluid secretion. Our experiments on the effects of the three natural aedeskinins on the fluid secretion rates and electrophysiology of isolated *Aedes* Malpighian tubules have confirmed an analogous study by Veenstra and colleagues with one glaring exception: In the laboratory of Veenstra, aedeskinin-II elicits electrophysiological effects but has no effect on fluid secretion (58). In our study we find that aedeskinin-II stimulates both fluid secretion and electrophysiology in a similar manner as aedeskinin-I and -III (Fig. 2).

The discrepancy between the results of our study and those of Veenstra may relate to our observation that occasionally Malpighian tubules do not respond to kinins at all. For example, in one series of experiments using the same hatch of mosquitoes, 2 out of 9 tubules did not respond to aedeskinin-I in the fluid secretion assay, and 5 of 19 Malpighian tubules did not respond in the electrophysiological assay (Fig. 5b,c). Similar observations can be made with aedeskinin-II and -III, as if the kinin receptor is not expressed in these Malpighian tubules.

Reviewing our data back to 1989 when we first started to study insect kinins (27), the observation of non-responding Malpighian tubules is rare, less than 1 % of all studies when tubules were obtained from adult mosquitoes 3-5 days old. However, that percentage can increase to 100% within some mosquito hatches (populations) where the responsiveness of the tubules to kinins is delayed for unknown reasons. Since these non-responding tubules are not the norm, we exclude them from the present study.

Non-responsiveness to kinins may have a developmental and/or nutritional causation. Tubules taken from imago mosquitoes shortly after eclosion may not express the kinin receptor. For example, the tubule shown in Fig. 5c had a normal, oscillating V_{bl} which did not change upon stimulation with aedeskinin-I. This tubule was taken from a mosquito two days after eclosion, when tubules from this hatch of mosquitoes did not respond to kinins. However, a few days later, hatch mates of this mosquito responded to kinin stimulation. As a first hypothesis, the time lag in receptor expression may reflect 1) the nutritional history of the mosquito in the larval stage, 2) the continued development of Malpighian tubules after eclosion, 3) the coupling of kinin receptor expression to reproduction, blood feeding, and/or other factors. For example, the quality of larval nutrition is known to affect ovarian development in *Aedes aegypti* (19, 25). Females with insufficient nutrition as larvae (due to a limited food supply or larval overcrowding) emerge as undersized ‘gono-inactive’ adults. These gono-inactive females

require additional feeding after emerging to develop their eggs to the previtellogenic resting stage of development. In contrast, females that are sufficiently nourished as larvae emerge as large 'gono-active' females with ovarian follicles capable of commencing vitellogenesis upon feeding on blood. Given that the diuretic responses triggered by the kinins would physiologically be most relevant to gono-active, blood-fed females, it is tantalizing to speculate that the expression of the kinin receptor in Malpighian tubules is linked to an endocrine and/or developmental axis of ovarian development.

Accordingly, development, nutrition, reproduction, blood-feeding and other physiological processes may all influence the expression of the kinin receptor in Malpighian tubules. It is therefore conceivable that Veenstra et al. (58) observed electrophysiological effects of all three aedeskinins when he used tubules from mature and well-developed mosquitoes, but failed to observe the effects of aedeskinin-II on fluid secretion in tubules isolated from developmentally or nutritionally compromised mosquitoes. Using mature mosquitoes in the present study, we have observed consistent effects of all three aedeskinins on both fluid secretion and electrophysiology (Fig. 2). Furthermore, the laboratory of Hagedorn reports that the injection of aedeskinin-II into the hemolymph of female *Aedes aegypti* increases urine production (17).

Aedeskinin-III duplicates the effects of leucokinin. Before the aedeskinins became available, our laboratory has studied the mechanism of action of the cockroach kinin, leucokinin-VIII, on Malpighian tubules of *Aedes aegypti*, where we made the first observation of the diuretic activity of the invertebrate family of kinins (27). We found that leucokinin-VIII increases the transepithelial secretion of water by increasing the transepithelial secretion of both NaCl and KCl (39). We have attributed the non-selective increase of both NaCl and KCl secretion to the activation of a large paracellular Cl⁻ conductance (5, 39, 59, 65).

Like leucokinin-VIII, aedeskinin-III increases the transepithelial secretion of NaCl and KCl, and both kinins significantly decrease the Na⁺ concentration and increase the K⁺ concentration in secreted fluid with no effect on the concentration of Cl⁻ (Fig. 3). Opposite effects on luminal Na⁺ and K⁺ concentrations (Fig. 3b) stem from the nearly complete transepithelial short-circuit caused by the large increase in the paracellular Cl⁻ conductance in the presence of kinins (5, 65). The transepithelial short circuit depolarizes the transepithelial voltage to values close to zero (39, 58) with the effect of 1) depolarizing the apical membrane voltage, and 2) hyperpolarizing the

basolateral membrane voltage (V_{bl}) of principal cells (Fig. 2). Since the K^+ conductance of the basolateral membrane of principal cells is four times greater than the Na^+ conductance (8), it follows that the hyperpolarization of V_{bl} increases the influx of K^+ from the peritubular medium to the cytoplasm of principal cells. As a result, the concentration of cytoplasmic K^+ is expected to rise, improving its competition with Na^+ for extrusion across the apical membrane into the tubule lumen. Consequently, the concentration of K^+ in the secreted fluid increases while that of Na^+ falls (Fig. 3b). The concentration of Cl^- in secreted fluid does not change because the luminal and peritubular Cl^- concentrations are already similar and high under control conditions (9, 20, 37, 61, 62). Accordingly, the kinin-induced increase in paracellular Cl^- conductance merely collapses the transepithelial voltage without a change in luminal or peritubular Cl^- concentration.

Voltage and resistance oscillations and the evidence for a role of Ca^{2+} . As shown in Figs. 2, 4 and 5, Malpighian tubules of *Aedes aegypti* often display oscillations of voltage and resistance under control conditions (6, 60, 63, 65, 67). Significantly, these oscillations are dependent on the Cl^- concentrations in the peritubular medium (6, 8). Oscillations of the transepithelial voltage that are greater than the simultaneous voltage oscillations of the basolateral and apical membranes (6, 47) point to the paracellular pathway as the source of oscillations. Indeed, the oscillations derive from spontaneous cyclical Cl^- conductance changes of the paracellular pathway (6). As the paracellular Cl^- conductance increases, the transepithelial voltage goes to values close to 0 mV (approaching transepithelial short circuit). Consequently, the basolateral membrane voltage (V_{bl}) hyperpolarizes and the apical membrane voltage (V_a) depolarizes (6, 47).

Studies in *Drosophila* Malpighian tubules have added importantly to our understanding of the spontaneous voltage and resistance oscillations observed in Malpighian tubules. Blumenthal has found that the biogenic amine tyramine is a potent stimulator of transepithelial Cl^- transport and a diuretic agent (14). The mechanism of action of tyramine, i.e. the sudden activation of a Ca^{2+} -dependent transepithelial Cl^- conductance and its kinin-like change in transepithelial voltage (depolarization) is strikingly similar to that of the kinin diuresis we have elucidated in *Aedes* Malpighian tubules (5, 39, 65-67). Moreover, low doses of tyramine (1-10 nM) induce oscillations of voltage and resistance in *Drosophila* Malpighian tubules (12, 13). What is

remarkable is that *Drosophila* Malpighian tubules generate their own tyramine from tyrosine (12), suggesting the autoregulation of fluid secretion in Malpighian tubules. The kinins and other diuretic agents as well as antidiuretic agents could have overriding influences on this autoregulation. Consistent with this hypothesis is the cross-desensitization of tyramine and leucokinin that Blumenthal reports in *Drosophila* Malpighian tubules (11, 13). Whether tyramine is responsible for the spontaneous oscillations in *Aedes* Malpighian tubules remains to be investigated.

Changes in intracellular $[Ca^{2+}]$ are likely to 1) mediate the spontaneous voltage oscillation, and 2) signal the electrophysiological effects of kinins in *Aedes* and perhaps also *Drosophila* Malpighian tubules (11, 67). First, the Ca^{2+} ionophore A23187 duplicates the effects of leucokinin in *Aedes* Malpighian tubules by lowering the transepithelial voltage and resistance, provided that Ca^{2+} is present in the extracellular Ringer bath (18, 67). Second, thapsigargin, the inhibitor of Ca^{2+} uptake by intracellular Ca^{2+} stores, duplicates the electrophysiological effects of leucokinin-VIII (67). Third, the voltage oscillations disappear in the presence of kinins (Fig. 2) which are known to activate nifedipine-sensitive Ca^{2+} channels in the basolateral membrane of principal cells (67). Fourth, analog 1460 II is the most potent synthetic kinin in an intracellular Ca^{2+} fluorescence assay (aequorin) of CHO-K1 cells expressing the recombinant kinin receptor of *Aedes aegypti* (52) and a potent activator of the electrophysiological response in *Aedes* Malpighian tubules (Fig. 4). Fifth, analog 1578 elicits a weak Ca^{2+} response in CHO-K1 cells expressing the kinin receptor of *Aedes aegypti* (52), and in the present study analog 1578 has no electrophysiological effect (Fig. 4). Furthermore, we found that analogs 1547, 1577, and 1578 progressively lose the ability to activate electrophysiological effects (Fig. 4), which is the rank order similar to that observed by Taneja-Bageshwar et al. using the intracellular Ca^{2+} bioassay in CHO-K1 cells expressing the kinin G protein-coupled receptor (52). Taken together, the above observations suggest Ca^{2+} mediates both the spontaneous voltage and resistance oscillations under control conditions and the electrophysiological effects of kinins. The signaling pathway(s) kinins use to increase fluid secretion are unknown.

One receptor, two or more signaling pathways. Whereas all three aedeskinins exert effects on both tubule electrophysiology and fluid secretion, not every synthetic kinin analog displays this twin effect. Analog 1708 has no effect on transepithelial fluid secretion but consistent effects on

tubule electrophysiology (Fig. 4). In contrast, analogs 1547, 1577, and 1578 consistently stimulate fluid secretion, but they lose the ability to activate electrophysiological effects with increasing length of the C-terminal pentapeptide (Fig. 4). Since *Drosophila* and *Aedes* express only one transcript of the single kinin receptor gene, which encodes a G protein-coupled receptor (GPCR) (41-43), the question arises as to how this single GPCR can give rise to the twin effects on fluid secretion and electrophysiology on the one hand, and to separate and independent effects on fluid secretion and electrophysiology on the other hand.

As a first hypothesis we consider ‘ligand-dependent selectivity of receptor signaling’ where a single receptor can give rise to more than one physiological response (Fig. 6). The phenomenon is also known as agonist-directed signaling, ligand-directed trafficking, conformation-specific agonism or functional selectivity; for reviews see (2, 48). Ligand-dependent signaling is particularly well known for GPCRs (48). For example, the single mammalian serotonin GPCR can activate 1) a phospholipase C-mediated increase in inositol phosphates, and/or 2) a phospholipase A₂-mediated release of arachadonic acid, depending on the ligand that binds to it (3, 49). Each ligand is thought to induce a distinct receptor conformation that targets a specific signaling pathway. For example, the binding of serotonin to the serotonin GPCR may induce one conformation that activates the inositol phosphate and arachadonic acid pathways equally, the binding of agonist (6)-1-(2,5-dimethoxy-4-iodophenyl)-2-amino propane may induce a different receptor conformation that preferentially activates the arachadonic acid pathway, and the binding of agonist 3-trifluoromethylphenyl-piperazine may trigger a receptor conformation that preferentially activates the inositol phosphate pathway (48). Similarly, the three aedeskinins, leucokinin, and analog 1460 II may activate equally those signaling pathways that increase rates of fluid secretion and induce electrophysiological changes (Figs. 2,6a). In the case of the other synthetic analogs, the amino acid substitutions produce conformations in the ligand that allow only partial binding to the receptor, thereby activating one physiological effect but not the other (Fig. 6a). Thus, the spatial modification found in analog 1708 may allow this analog to interact with the region of the receptor which activates the electrophysiological response while preventing interactions with the receptor region associated fluid secretion (Fig. 6a). In contrast, the spatial modifications of analog 1578 may allow the interaction with the receptor binding sites associated with the activation of fluid secretion, but prevent the interaction with binding sites that signal electrophysiological effects (Fig. 6a).

As a second hypothesis, we propose that the single kinin GPCR may control the access of ligands by way of glycosylation (Fig. 6b). GPCRs are typically glycoproteins with the glycosylation sites existing within the extracellular N-terminal region (46). The consensus sequence Asn-X-Ser-/Thr provides for N-linked glycosylations. Although *Drosophila* Malpighian tubules express a single transcript encoding a kinin GPCR (consistent with one receptor), two immunochemical forms of the drosokinin GPCR are detected: one is N-glycosylated and the other is not (43). Likewise, Malpighian tubules of the *Anopheles* mosquito express two analogous immunochemical forms of the kinin GPCR (44). If we assume that *Aedes* Malpighian tubules also express two varieties of the kinin GPCR, R₁ and R₂, then the glycosylated receptor may signal one physiological event and the non-glycosylated receptor may signal the other. Thus, the three aedeskinins, leucokinin-VIII and 1460 II may bind to both GPCRs, thereby triggering the twin physiological response of fluid secretion and tubule electrophysiology (Fig. 6b). However, in the case of analogs 1708 and 1578, the chemical modifications to the peptides may favor binding to the glycosylated form of the GPCR over the non-glycosylated form (or vice versa), resulting in one physiological response that dominates over the other. Glycosylation-dependent ligand binding of GPCRs and signaling has been observed. The glycosylation can influence 1) receptor-receptor self-association (24), 2) stabilization of the GPCR in the plasma membrane (53), 3) folding of the GPCR into a high affinity conformation (68), 4) membrane trafficking and surface expression of the GPCR (15, 40), and 5) ligand binding and the ensuing signal transduction response (1, 35).

The phenomena of agonist-directed or glycosylation-dependent signaling now sheds light on an observation that has been enigmatic to us for the past 21 years: Leucokinin-VIII produces different dose response curves of its effects on 1) the transepithelial voltage, and 2) rates of fluid secretion in *Aedes* Malpighian tubules (27). The voltage dose response curve is steep with an EC₅₀ of 2.4×10^{-9} M, whereas the fluid secretion dose response curve is shallow with an EC₅₀ of 3.0×10^{-8} M (27), similar to that observed in the present study using aedeskinin-III (Fig. 3a). Veenstra (41, 58) made similar observations finding that the aedeskinins have different threshold concentrations when assayed for the depolarizing activity of the transepithelial voltage and also different potencies on fluid secretion. Veenstra et al. suggested more than one type of kinin receptor to account for the differences observed in electrophysiological and fluid secretion assays. The present study suggests agonist-directed signaling of kinins, or alternatively, a

differentially glycosylated receptor that can activate electrophysiological or fluid secretion responses.

Perspectives and Significance. In the present study we have used two bioassays - electrophysiology and fluid secretion - to probe kinin/receptor interactions in Malpighian tubules of the yellow fever mosquito *Aedes aegypti*. We have observed that the three natural aedeskinins activate both physiological responses. In contrast, synthetic kinin analogs containing spatial modifications in the critical pentapeptide core region may trigger just one or both effect(s). Separate and independent effects on electrophysiology and fluid secretion may reflect ligand-directed signaling and/or glycosylation-dependent functional states of the single kinin receptor expressed in *Aedes* Malpighian tubules. Separate effects of kinin analogs on electrophysiology and fluid secretion suggest that natural kinins may be physiologically more diverse than previously assumed. For example, dose-dependent effects on tubule electrophysiology without an effect on fluid secretion may indicate the renal response to a purely ionic challenge, whereas effects on fluid secretion without an effect on electrophysiology may indicate the renal (electroneutral) response to a volume load. More than one bioassay should be consulted to fully evaluate the structure/function relations of insect kinins and their G protein-coupled receptors. The reliance on only one bioassay (as in high-throughput screens of voltage effects or changes in intracellular Ca^{2+} concentrations) would have excluded analog 1578 as a potential diuretic agent. By incorporating the Ramsay fluid secretion assay, we have proven otherwise.

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Appendix

On changes in voltage and fluid secretion.

In response to a reviewer's inquiry about how voltage effects can be independent from effects on ion transport and fluid secretion in Malpighian tubules, it is instructive to compare the quantities of ions (charge) needed to change a voltage with the quantities of ions secreted across

the epithelium into the tubule lumen. Figure 3 illustrates that in the presence of aedeskinin-III the tubule secretes cations or anions at a rate of approximately 165 pmol/min. The secretion is mediated by the 1.5 mm tubule segment suspended in the Ringer droplet of the Ramsay fluid secretion assay (Fig. 1a). Since the average electrical diameter of the *Aedes* Malpighian tubule determined by cable analysis is about 22 μm (38), the transepithelial cation or anion secretion can be normalized to an epithelial surface area, i.e. of 2.7×10^{-9} moles/s-cm². Applying the Faraday constant yields an electrical equivalent of 2.6×10^{-4} coul/sec-cm², or 260 $\mu\text{A}/\text{cm}^2$.

The electrical capacitance of epithelia is approximately 3.5 $\mu\text{F}/\text{cm}^2$ (33). The quantity of charge q is the product of capacitance (C) and voltage (V). Accordingly, the kinin-induced depolarization of the transepithelial voltage of *Aedes* Malpighian tubule from 59 mV to 6 mV (39) requires a charge redistribution of only 1.9×10^{-7} coul/cm² which takes only 0.72 ms at the transepithelial charge secretion rate of 2.6×10^{-4} coul/sec-cm². In that short time the tubule secretes a volume of only 1.2×10^{-14} liter of fluid, far too small for detection in the Ramsay fluid secretion assay. The above calculations illustrate that a change of voltage requires very little ion flux. Accordingly, a change in voltage may not reflect a significant change in ion and fluid secretion.

The kinin-induced drop in transepithelial voltage from 59 mV to 6 mV together with the drop in transepithelial resistance from 58 Ωcm^2 to 10 Ωcm^2 (39) indicates the switch from a moderately tight epithelium to a leaky epithelium. At the extremes, leaky epithelia are specialized for massive transepithelial transport of solute and water (often in isosmotic proportions as in renal proximal tubules), and tight epithelia (with low rates of transepithelial transport) are specialized for storage functions (such as the urinary bladder). In tight epithelia, changes in transepithelial ion transport are reflected in changes in voltage and resistance; however, in leaky epithelia large changes in transepithelial ion transport may take place with little effect on voltage and resistance, because leaky epithelia operate near the short-circuit condition (where ‘short-circuit’ is the condition of zero voltage).

Table 1. Kinins and synthetic analogs (28).

PEPTIDE	Primary Structure	Reference
Conserved kinin sequence	Phe-X²-X³-Trp-Gly-NH₂	(56)
Leucokinin-VIII	Gly-Ala-Asp- Phe-Tyr-Ser-Trp-Gly-NH₂	(30)
Aedeskinin I	Asn-Ser-Lys-Tyr-Val-Ser-Lys-Gln-Lys- Phe-Tyr-Ser-Trp-Gly-NH₂	(57)
Aedeskinin II	Asn-Pro- Phe-His-Ala-Trp-Gly-NH₂	(57)
Aedeskinin III	Asn-Asn-Pro-Asn-Val- Phe-Tyr-Pro-Trp-Gly-NH₂	(57)
1460 II	Ac-Arg- Phe-Phe-[β³Pro]-Trp-Gly-NH₂	(52)
1547	Phe-Phe-Phe-Ser-Trp-Gly-NH₂	(51)
1577	Ac-Arg-[β ³ Phe]- Phe-Phe-[β³Pro]-Trp-Gly-NH₂	(52)
1578	Ac-Arg- Phe-[β³Phe-β³Pro]-Trp-Gly-NH₂	(52)
1708 , K-Aib-4	Ac-Arg-[β ³ Phe]- Phe-Phe-[Aib]-Trp-Gly-NH₂	(50)

Bold print identifies the critical pentapeptide sequence for activity in natural kinins; Ac, acetyl group; β³, beta-3 amino acid (with methylene group preceding the carbonyl group in the backbone); X² can be His, Asn, Ser, Phe or Tyr; X³ can be Ser, Pro or Ala; Aib, α-amino-isobutyric acid (31, 52).

Figure Legends

Fig. 1. Experimental methods: a) the Ramsay fluid secretion assay, and b) two-electrode voltage clamp method (TEVC) for measurements of the basolateral membrane voltage (V_{bl}) and the input resistance (R_{in}) of a principal cell (PC) in an isolated Malpighian tubule, modified from (64). The Ramsay assay requires a minimum of 5 to 10 min before effects of kinins added to the peritubular bath are observed. The TEVC method yields immediate data of voltage and resistance. A scanning electron micrograph of a Malpighian tubule of a female mosquito (*Aedes aegypti*) is shown in panel b.

Fig. 2. The effect of leucokinin-VIII and aedeskinins-I, -II, -III on the rate of transepithelial fluid secretion and the basolateral membrane voltage and input resistance of principal cells in isolated Malpighian tubules of female *Aedes aegypti*. All kinins were used at a concentration of 10^{-6} M. Data are mean \pm SE with the number of paired tubule experiments in parenthesis. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ paired t-test. Data of the effect of leucokinin-VIII on fluid secretion were taken from a previous study (39) for comparison with the effect of aedeskinins.

Fig. 3. a) Dose-response curve for the effects of aedeskinin-III on transepithelial fluid secretion in isolated Malpighian tubules of *Aedes aegypti*. The effect of aedeskinin-III (10^{-6} M) on the concentrations of ions in secreted fluid and the rates of transepithelial ion secretion are shown in panels b) and c) respectively. Open and shaded bars represent mean \pm SE with the number of paired tubule experiments in parentheses. * $p < 0.02$, ** $p < 0.01$, *** $p < 0.001$, paired t-test.

Fig. 4. The effect of synthetic kinin analogs on the rate of transepithelial fluid secretion and the basolateral membrane voltage and input resistance of principal cells in isolated Malpighian tubules of female *Aedes aegypti*. Kinin analogs were used at a concentration of 10^{-6} M. Analog 1708 stimulated fluid secretion in some tubules and inhibited it in other tubules, such that on average in 29 tubules, 1708 had no significant effect on fluid secretion. Open and shaded bars represent means \pm SE with the number of tubule experiments in parentheses; * $p < 0.05$, ** $p < 0.01$, paired t-test.

Fig. 5. Representative electrophysiological responses of Malpighian tubules of *Aedes aegypti* to stimulation with kinins and analogs. a) sham Ringer bath change at a Ringer flow rate of 5 ml/min; b) tubule with non-oscillating, high basolateral membrane voltage (V_{bl}); c) tubule with the usual oscillating basolateral membrane voltage (V_{bl}); d) aedeskinin receptor desensitization; e) lack of effect of kinin analog 1578 on tubule electrophysiology. AK, aedeskinin.

Fig. 6. Models of ligand/receptor interactions. a) Ligand-dependent signaling via a single receptor; (model adopted from (2). b) Glycosylation-dependent signaling, where R_1 and R_2 represent glycosylated and non-glycosylated forms of the same receptor.

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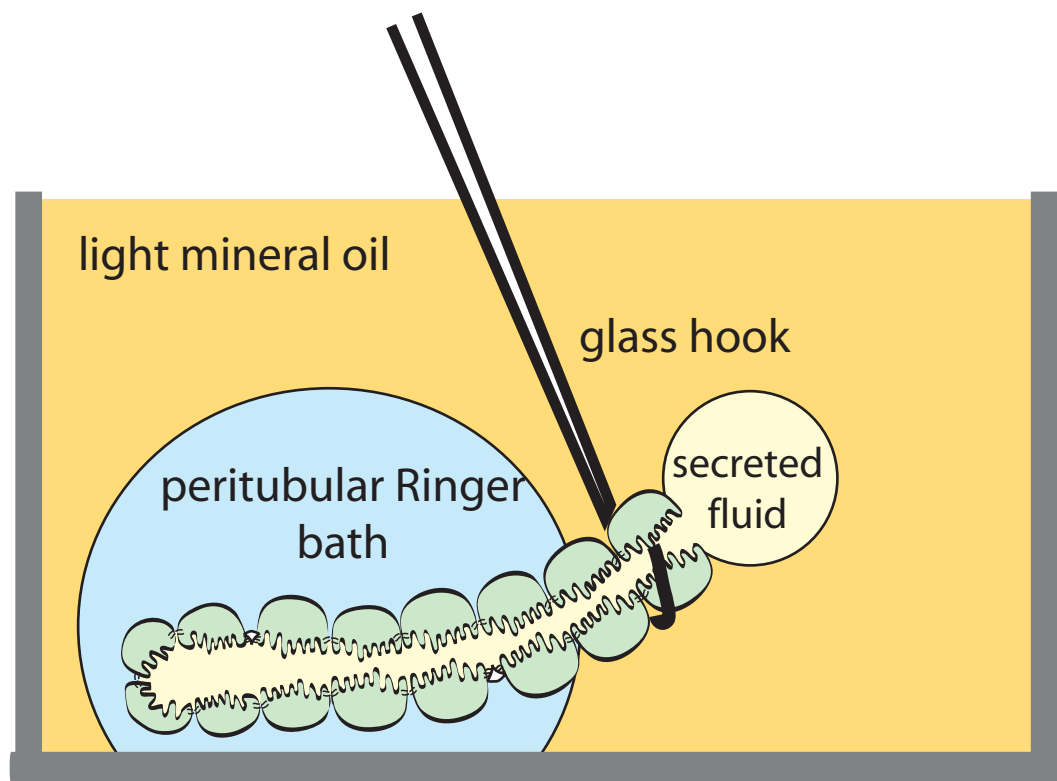
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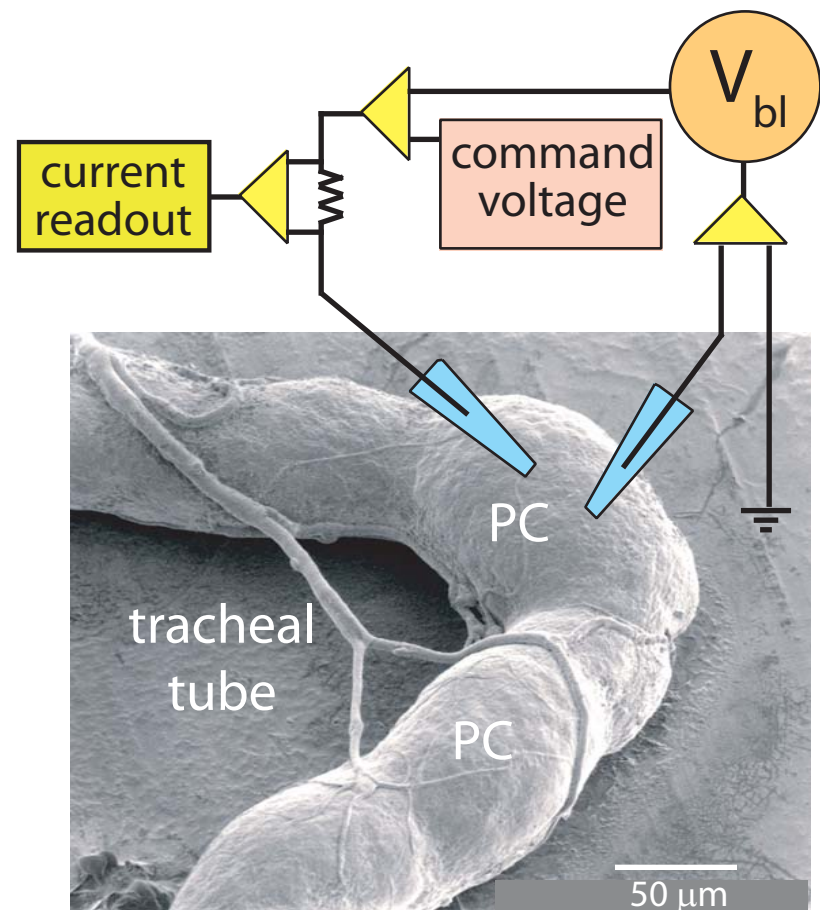
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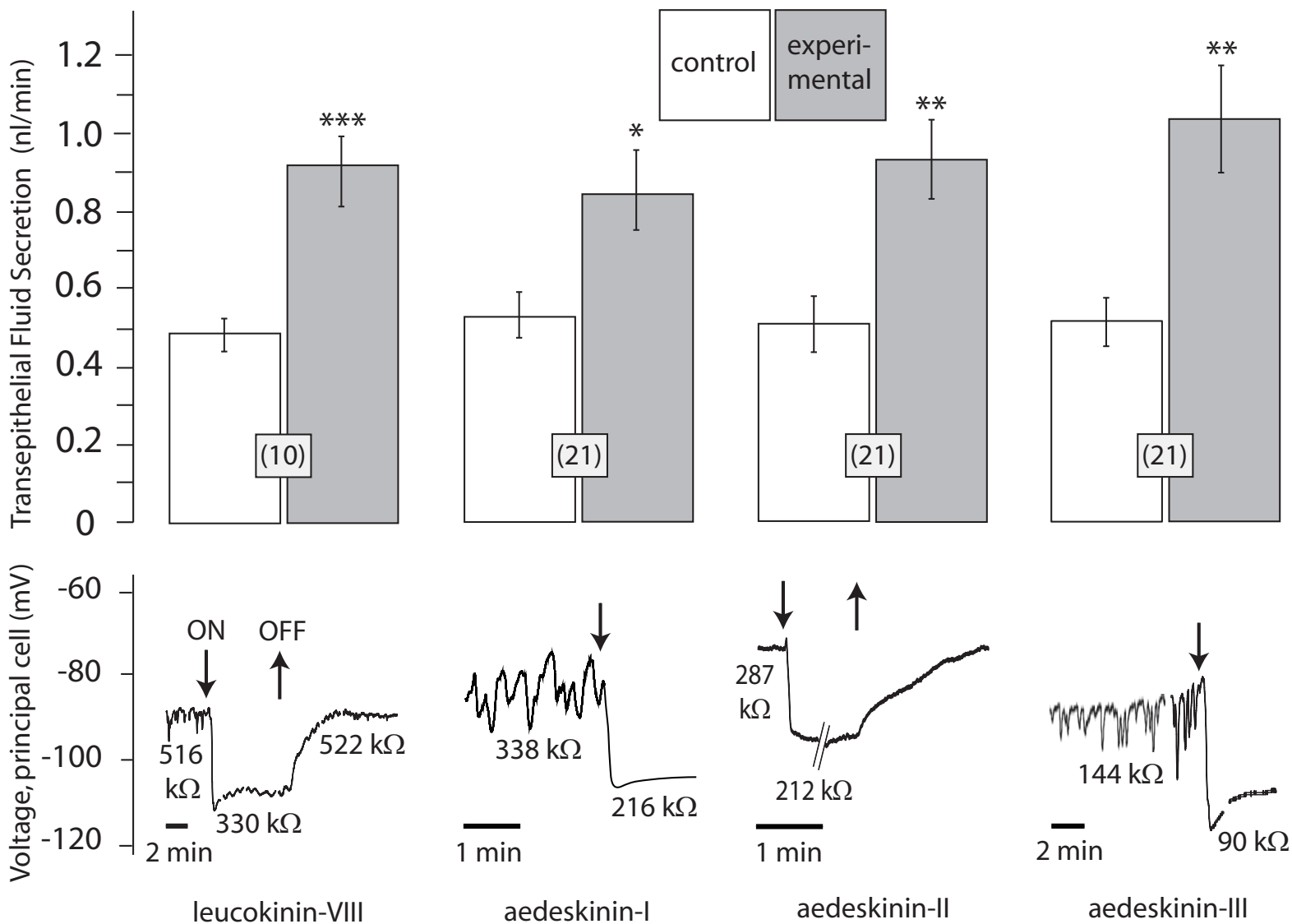
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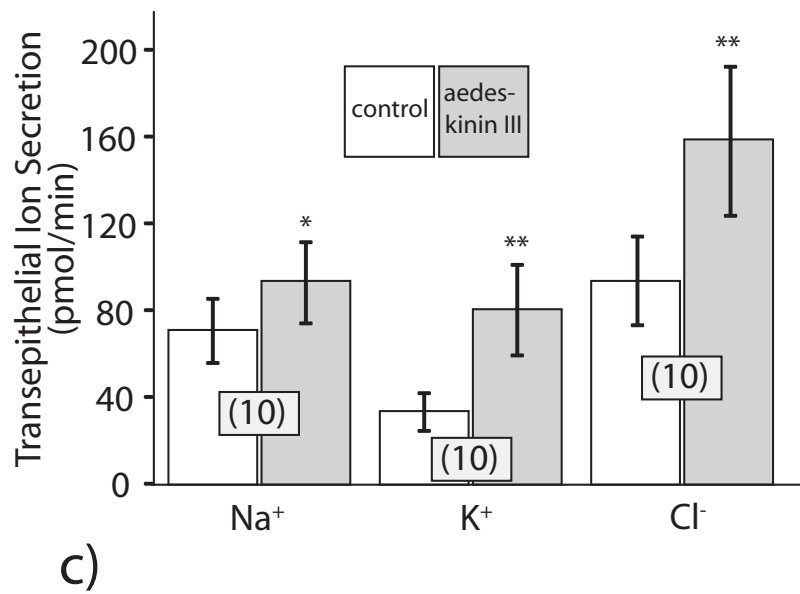
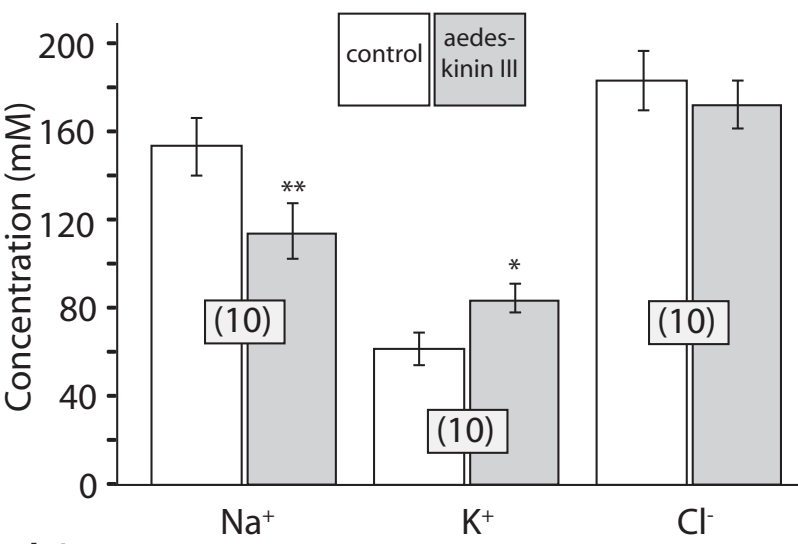
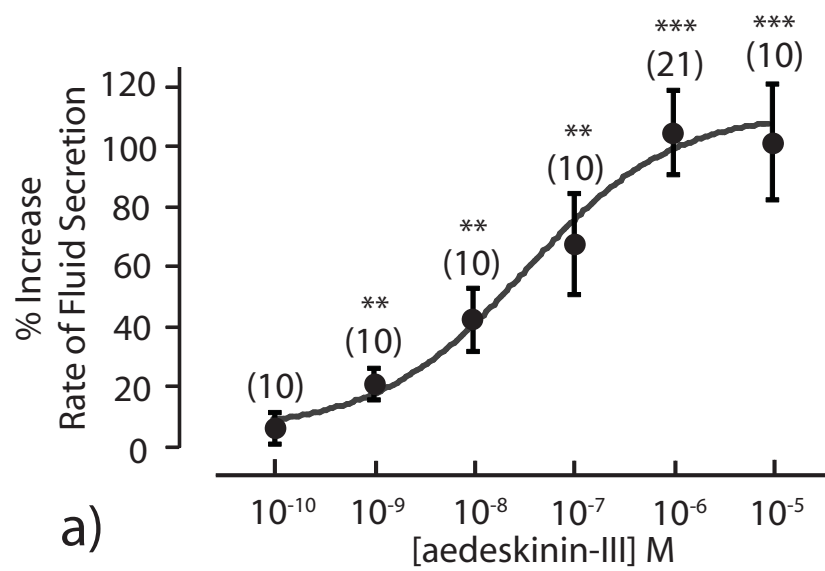


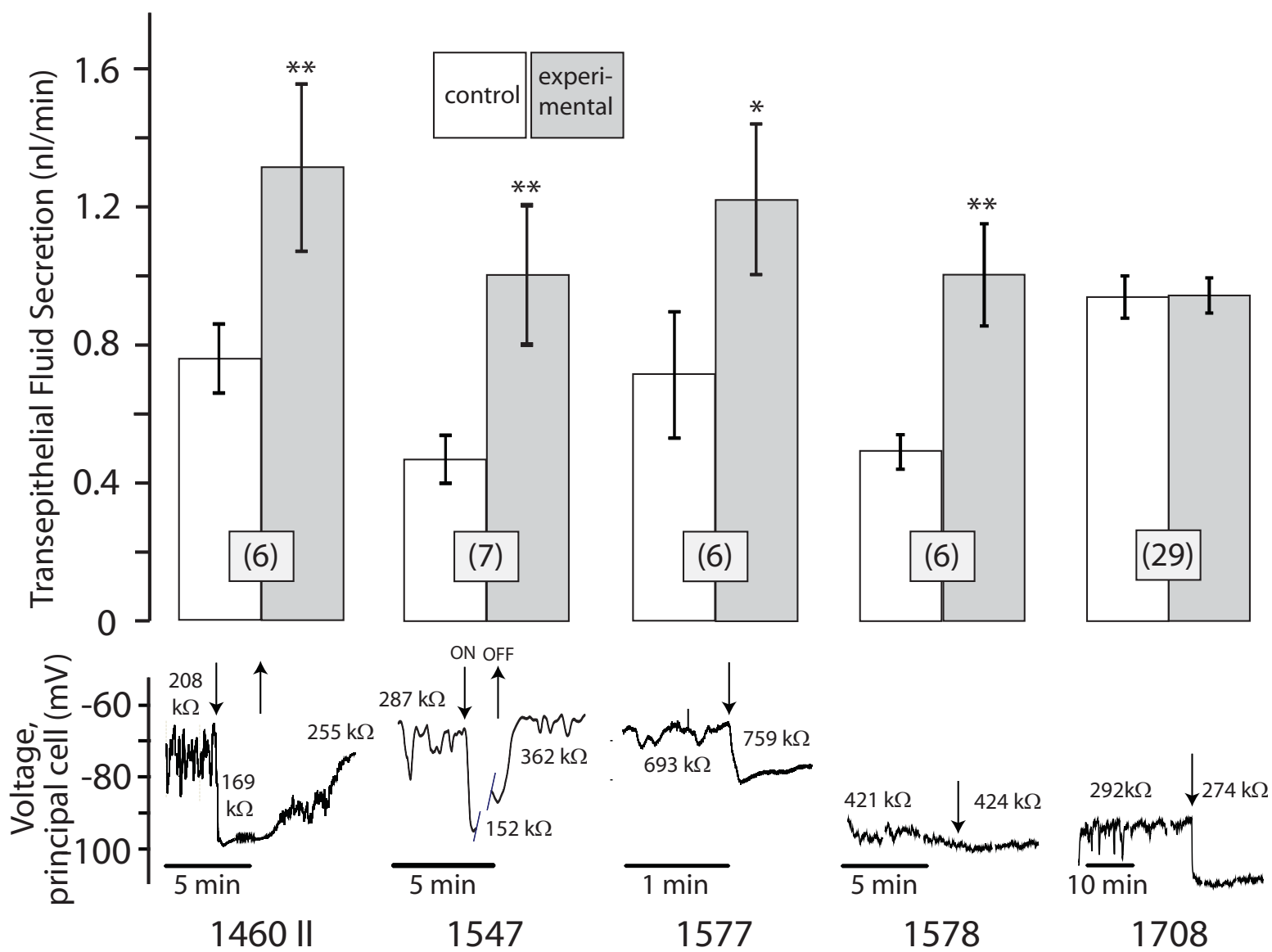
a)



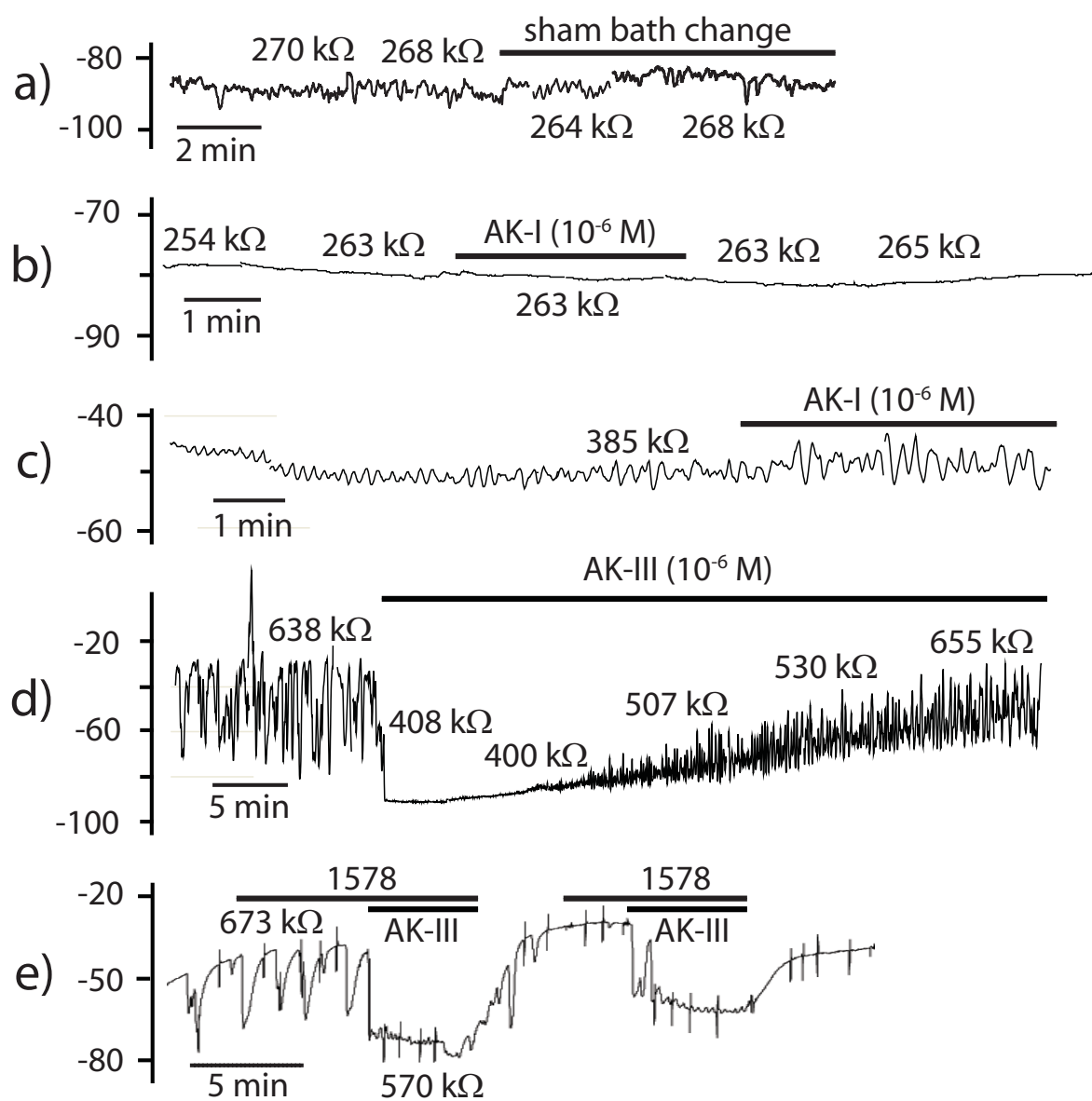
b)



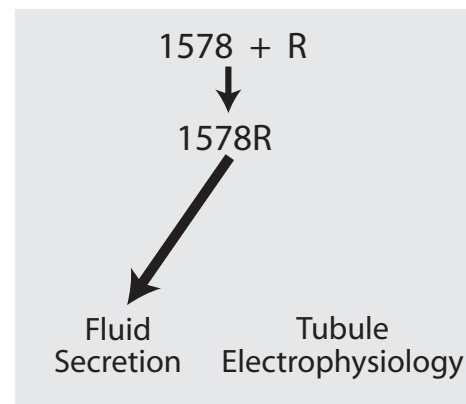
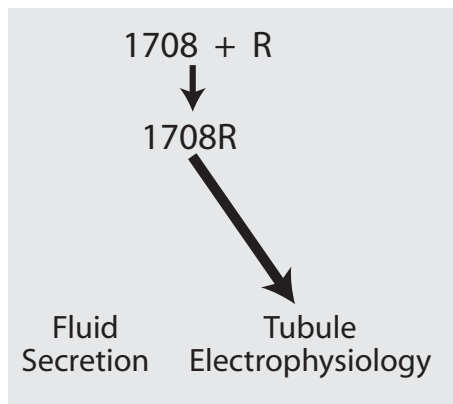
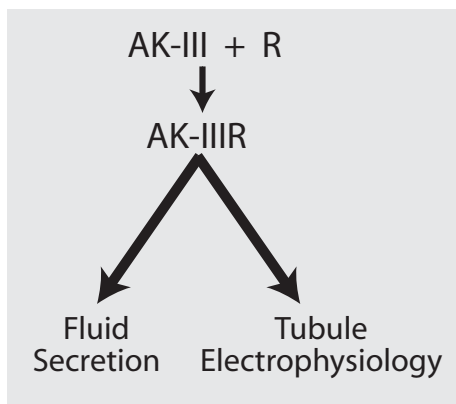




Voltage (V_{bl}), principal cell (mV)



a)



b)

